Fisheries Research 202 (2018) 38–48

Contents lists available at ScienceDirect

Fisheries Research

journal homepage: www.elsevier.com/locate/fishres

Full length article

Species-specific Real Time-PCR primers/probe systems to identify fish parasites of the genera *Anisakis*, *Pseudoterranova* and *Hysterothylacium* (Nematoda: Ascaridoidea)

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ARTICLE INFO

Keywords:
Real Time-PCR
mtDNA *cox2*
Anisakis
Pseudoterranova
Hysterothylacium
Ascaridoid identification

ABSTRACT

Ascaridoid nematodes belonging to the genera *Anisakis* and *Pseudoterranova* are heteroxenous parasites, involving marine mammals as definitive hosts in their life-cycles, whereas crustaceans (krill), fish and squids acting as intermediate/paratenic hosts. These parasites are considered among the most important biological hazards present in "seafood" products. Indeed, larval stages of the *Anisakis* and *Pseudoterranova* have been reported as etiological agents of human infections (anisakidosis). We developed a primers/probe system for the identification of five species of anisakid nematodes belonging to the genera *Anisakis* (i.e. *A. pegreffii* and *A. simplex* (s. s.)), and *Pseudoterranova* (i.e. *P. decipiens* (s. s.), *P. krabbei* and *P. bulbosa)* to be used in a real time polymerase chain reaction (RT-PCR) with specific primers based on the mtDNA *cox2* gene. Because those anisakid species could be also found in co-infection in some fish species with the raphidascarid nematode *Hysterothylacium aduncum*, a species-specific primer probe system to be used in RT-PCR for this nematode species was also developed.

The detection limit and specificity of the primer/probe systems were evaluated for each of the six nematode species. Singleplex and multiplex RT-PCR protocols were defined and tested. The detection limit of the nematode species tissue was lower than 0.0006 ng/μl. Efficiency (*E*) of primers/probe systems developed was carried out by standard curve; *E* value varied between 2.015 and 2.11, with respect to a perfect reaction efficiency value of $E = 2$. Considering the sensibility and quantitative nature of the assays, the new primers/probe system may represent a useful tool for future basic and applied research that focuses on the identification of *Anisakis* spp., *Pseudoterranova* spp. and *H. aduncum* larvae in fish, even in co-infections, with a potential for application in fish farming, fish processing industries, fish markets, and food producers.

1. Introduction

Ascaridoid nematodes belonging to the genera *Anisakis* and *Pseudoterranova* are heteroxenous parasites, involving marine mammals as definitive hosts, whereas various planktonic or semi-planktonic crustaceans (e.g., krill), fish and squids acting as intermediate/paratenic hosts in their life-cycles. These parasites are currently considered among the most important biological hazards present in "seafood" products (EFSA Panel on Biological Hazards (BIOHAZ), 2010). Indeed, larval stages of *Anisakis* spp. and *Pseudoterranova* spp. are the causative agents of human anisakiasis and pseudoterranoviasis, respectively (Ishikura and Kikuchi, 1990; Daschner et al., 2012; Nieuwenhuizen, 2016). Among the species of *Anisakis* so far genetically characterized (Mattiucci and Nascetti, 2008; Mattiucci et al., 2009), only *A. pegreffii* and *A. simplex* (s. s.) have been documented to cause infections in humans (D'Amelio et al., 1999; Umehara et al., 2007; Fumarola et al., 2009; Moschella et al., 2004; Mattiucci et al., 2011, 2013; Qin et al., 2013; Arai et al., 2014; Lim et al., 2015). Among the species of the genus *Pseudoterranova* so far genetically characterized (Mattiucci and Nascetti, 2008; Timi et al., 2014), human cases of pseudoterranoviasis are reported as inflicted by *P. azarasi* (Arizono et al., 2011) and *P. decipiens* (s. s.) (Cavallero et al., 2016). The human disease is the result

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http://dx.doi.org/10.1016/j.fishres.2017.07.015
Received 15 February 2017; Received in revised form 12 July 2017; Accepted 14 July 2017
Available online 29 July 2017

of the accidental ingestion of live third stage larvae present in raw or undercooked infected seafood, particularly in the fillets of various commercially important fish species (Levsen et al., 2017, in press). Thus, larval nematodes of the genera *Anisakis* and *Pseudoterranova* are of economic concern, due to public health implications and undesirable effects on the marketability of fish products (Levsen et al., 2017, in press). Currently, one between the most prevalent species of nematodes belonging to the Family Raphidascarididae infecting fish in European waters, at both larval and adult stage, is *Hysterothylacum aduncum*. Its adult stage infects gastrointestinal tract of teleost fish species; whereas, their third stage larvae can be found in the mesentery and viscera of benthic and pelagic fish hosts (Køie, 1993; Klimpel and Rückert, 2005; Levsen et al., 2016; Cipriani et al., 2017). However, so far, larval stages of *H. aduncum* have never been found to infect fish musculature (Levsen et al., 2016; Cipriani et al., 2017). Additionally, despite the fact that accidental ingestion of *H. aduncum* was recently found in a human patient with epigastralgia (González-Amores et al., 2015), the zoonotic role of species of *Hysterothylacium* remains uncertain. While several PCR-DNA protocols have been developed for the diagnosis of the species of the genus *Anisakis* (D'Amelio et al., 2000; Nadler et al., 2005; Valentini et al., 2006; Mattiucci et al., 2014, 2016) and *Pseudoterranova* (Zhu et al., 2002; Timi et al., 2014), RT-PCR species-specific probes for *Anisakis* spp. and *Pseudoterranova* spp. have not yet been fully proposed. Although a primer-probe system on a RT-PCR assay based on the mtDNA *cox2* gene sequence (Lopez and Pardo, 2010) was previously attempted on species of the genus *Anisakis*, however the method was not able to discriminate between the two zoonotic species *A. simplex* (s. s.) and *A. pegreffii*. Another primer-probe system was based on the ITS region of the rDNA, allowing the distinction between *A. simplex* (s. s.) and *A. pegreffii* (Mossali et al., 2010). Based on the same method, Cavallero et al. (2014) proposed a HRM (high resolution melt) to identify two species (i.e. *A. pegreffii* and *A. simplex* (s. s.)) of the genus *Anisakis*. Similarly, the ITS region of the rDNA was used to detect anisakids in the seafood products (Espiñeira et al., 2010), but the method was not able to identify the larvae at their species level. Furthermore, a real-time PCR method for the detection *in situ* was proposed to amplify a region of the ITS-2 rDNA only for the species *Anisakis pegreffii* (Fang et al., 2011). A further probe based on the mtDNA *cox1* was also proposed (Herrero et al., 2011); however, it was not able to distinguishing different species of anisakid nematodes. Finally, a RT-PCR assay based on primers/probe system to detect raphidascarid species belonging to the genus *Hysterothylacium* has never been carried out. L3 larval stages of *H. aduncum* can be found in co-infection with *Anisakis* larvae in viscera and mesenteries of commercially relevant fish species, thus their rapid and specific identification assume relevance for the assessment of sea-food quality and human safety.

The aim of the present work was to develop a species-specific primer/probe systems to be used in RT-PCR based on the mtDNA *cox2* gene, for the identification of ascaridoid species belonging to the genera *Anisakis*, *Pseudoterranova* and *Hysterothylacium*. Species belonging to these genera frequently occur, even in co-infection, as larvae in commercially important marine fish species from European waters (Levsen et al., 2017, in press).

2. Materials and methods

The number of specimens of the considered parasites- i.e. *A. pegreffii, A. simplex* (s. s.), *P. decipiens* (s. s.), *P. krabbei*, *P. bulbosa*, and *H. aduncum,* used for the development of specific primers and probes are reported in Table 1. Data regarding parasites host species and collecting localities are included in Table 1. The specimens were stored in fridge at −80 °C and/or in ethanol at 70%, until DNA extraction. Part of the data for the fish and nematodes was collected and reported in a Biobanking platform specifically designed within the PARASITE project (González et al., 2017, in press).

2.1. Direct DNA sequencing for the identification of ascaridoids used for RT-PCR probes development

As first step, genomic DNA – to be then used for the RT-PCR development – was extracted from each ascaridoid nematode (Table 1) using the "DNeasy blood and tissue" kit (Qiagen). Direct sequences analysis was then performed for their identification to species level. In particular, the sequences analysis of mtDNA *cox2* and EF1 α-1 nDNA genes were used to identify specimens belonging to the species *A. pegreffii* and *A. simplex* (s. s). Analogously, sequences analysis of the ITS1 and ITS2 region of the rDNA and mtDNA *cox2* genes was used to identify the specimens of *Pseudoterranova decipiens* (s. s.), *P. krabbei* and *P. bulbosa* and for the raphidascarid *H. aduncum*.

The mitochondrial cytochrome C oxidase subunit-2 (*cox2*) gene was amplified in the larval ascaridoid species – here considered – using the primers 211F (5′-ttt tct agt tat ata gat tgr tty at-3′) and 210R (5′-cac caa ctc tta aaa tta tc-3′) (Nadler and Hudspeth, 2000; Valentini et al., 2006; Timi et al., 2014). The PCR (polymerase chain reaction) was carried out using the following conditions: 94 °C for 3 min (initial denaturation), followed by 34 cycles at 94 °C for 30 s (denaturation), 46 °C for 60 s (annealing), 72 °C for 90 s (extension), followed by post amplification at 72 °C for 10 min.

PCR (polymerase chain reaction) amplification of EF1 α-1 nDNA gene of the two species of the genus *Anisakis,* i.e. *A. pegreffii* and *A. simplex* (s. s.), was performed using the primers EF-F (5′-TCC TCA AGC GTT GTT ATC TGT T-3′) and EF-R (5′-AGT TTT GCC ACT AGC GGT TCC-3′) (Mattiucci et al., 2016). PCRs were performed in 25 µl volume, containing 0.5 µl of each primer 10 mM, 2.5 µl of $MgCl_2$ 25 mM (Promega), 1.5 µl of 5× buffer (Promega), DMSO 0.08 mM, 0.5 µl of dNTPs 10 mM (Promega), 5 U of Go-*Taq* Polymerase (Promega), 2 µl (~2 ng/µl) of total DNA and sterilized analytical grade water to reach the final volume. PCR temperature conditions were the following: 94 °C for 3 min, followed by 35 cycles at 94 °C for 45 s, 58 °C for 40 s, 72 °C for 1 min, and followed by post amplification at 72 °C for 10 min (Mattiucci et al., 2016).

For the sequencing of the internal transcribed spacer (ITS rDNA) region of *Pseudoterranova* spp. and *H. aduncum* species, PCR amplification was performed using the primers NC5 (5′-GTA GGT GAA CCT GCG GAA GGA TCA TT-3′) and NC2 (5′-TTA GTT TCT TTT CCT CCG CT-3′) (Zhu et al., 2002; Timi et al., 2014). PCR amplification conditions were the following: 95 °C for 10 min, followed by 30 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 75 s, and a final elongation step at 72 °C for 7 min.

The PCR products were run on 1.0% agarose gel with 1.5 µl of Gelred, and visualized by shortwave ultraviolet light illumination, in order to verify the correct length of new fragments, using a 100 bp ladder as template.

2.2. Design of species-specific primers and probes to be used in RT-PCR

Sequences of the mtDNA *cox2* gene of the following anisakid species: *Anisakis pegreffii* (N = 20) (629 bp), *A. simplex* (s. s.) (N = 20) (629 bp), *Pseudoterranova krabbei* (N = 10) (519 bp), *P. bulbosa* (N = 10) (519 bp) and, finally, *P. decipiens* (s. s.) (N = 5) (519 bp), obtained from our previous studies (Mattiucci et al., 2012, 2013, 2014; Timi et al., 2014), were considered to design primers and probe to be used in RT-PCR. In addition, mtDNA *cox2* sequences (N = 10) (593 bp) of *H. aduncum* larvae were added to the dataset alignment. Sequences were first aligned using ClustalX software (Thompson et al., 1997) to design, by eye, specific internal primers and probes to be used for the Real-time PCR for each anisakid species considered. Primers/probes combinations were then confirmed using Primer3 on-line software (http://biotools.umassmed.edu/bioapps/primer3). The sequences alignment at the mtDNA *cox2* gene in all the considered nematodes species are shown in Fig. 1, in comparison with sequences of mtDNA *cox2* of nematodes obtained from fish species of commercial importance

Table 1
Number of specimens belonging to species of the genera *Anisakis*, *Pseudoterranova* and *Hysterothylacium* (previously identified by other genetic/molecular methods), used for the RT-PCR primers/probes systems development. Data on host species and sampling areas are also reported. APE: *Anisakis pegreffii*; ASS: *A. simplex* (s. s.); PDS: *Pseudoterranova decipiens* (s. s.); PDK: *P. krabbei*; PDB: *P. bulbosa*, HYS: *Hysterothylacium aduncum*.

Host species	Sampling area	Life-history stage	Nematode species					
			APE	ASS	PDS	PDK	PDB	HYS
Merluccius merluccius	Adriatic Sea	L3	22	–	–	–	–	–
Scomber scombrus	"	L3	15	–	–	–	–	–
Engraulis encrasicolus	Tyrrhenian Sea	L3	4	–	–	–	–	–
Merluccius merluccius	"	L3	16	–	–	–	–	–
Clupea harengus	Norwegian Sea	L3	–	35	–	–	–	–
Gadus morhua	"	L3	–	–	–	–	11	–
Hippoglossoides platessoides	"	L3	–	–	2	–	–	–
Scomber scombrus	"	L3	–	18	5	–	–	–
Mallotus villosus	"	L3	–	–	–	–	–	4
Gadus morhua	North Sea	L3	–	–	3	9	2	1
Pleuronectes platessa	"	L3	–	–	–	–	–	2
Pollachius virens		L3	–	–	–	–	–	11
Scomber scombrus	"	L3	–	–	2	–	–	1
Phoca vitulina	"	A	–	–	3	–	–	–
Halichoerus grypus	"	A	–	–	3	3	5	–
Erignathus barbatus	"	A	–	–	–	–	5	–

available in GenBank. The species-specific primers/probes for each target nematode species are reported in Table 2.

In order to exclude the amplification of fish host DNA, the sequences of mtDNA *cox2* of fish species of commercial importance retrievable from GenBank, and known to be naturally infected by the considered ascaridoid species (Mattiucci and Nascetti, 2008; Levsen et al., 2017, in press), were aligned with the primers 211F and 210R, and with the mtDNA *cox2* sequences of the present ascaridoid species. No match was found between the mtDNA *cox2* of the fish species and the specific amplification primers selected for the considered nematode species.

2.3. Singleplex RT-PCR protocol optimization

The extracted DNA from each specimen belonging to the considered nematode species, previously identified by means of direct sequencing of the mtDNA *cox2*, EF1 α-1 nDNA, and ITS region of the rDNA, was used in singleplex reactions in order to verify the specificity of each single primers/probe system for the those parasite species. At first, extracted parasite DNA was used to test the species-specific primers/probes separately, using pure DNA from each specimen. All the tests were then performed in triplicate for each nematode species, including a negative control. Primers and probes sequences, length of the fragment obtained, fluorophore labelling, and concentration of primers and probes used in the RT-PCR experiments, are reported in Table 2. Different concentrations of those primers and probes designed for the six parasite species were tested by several experiments, in order to optimize the PCR reaction mix; 0.1, 0.2 and 0.3 mM for primers, and 0.2, 0.3, 0.4 and 0.5 mM for probes concentrations were tested for PCR-mix optimization. Finally, the following standardization of the same PCR conditions, for all the primers and probes, was obtained: the PCR assays were performed in a total volume of 15 µl, containing 7.5 µl of LC FS DNA Hy.Probe MASTER (2x) (Roche®) and 1.5 µl (2–5 ng/µl) of DNA template; to adjust the final volume, serial concentrations of primers and probes, and sterilized analytical grade water, were used. The thermal cycling protocol was tested using a range of annealing temperature between 55 and 65 °C, in order to target the optimal one. Reactions were carried out as following: 95 °C for 10 min, followed by 40 cycles at 95 °C for 10 s, 55–65 °C for 30 s, 72 °C for 1 s, followed by a cooling step at 40 °C for 30 s. The reactions were run in a Light Cycler 480 II System (Roche®) Real Time PCR instrument, using LightCycler 480 Multiwell Plate 96 white (Roche®) and LightCycler 480 Sealing Foil (Roche®).

In addition, in order to verify the specificity of the primers and probe designed for those nematode species, the genomic DNA of the host fish species was tested at different conditions. Each one was analyzed in triplicate, at different amplifications, to test for a possible cross-reactivity with the parasites DNA.

2.4. Multiplex RT-PCR protocol optimization

In a second step, the extracted DNA from those ascaridoid specimens previously identified to their species level, was used in multiplex RT-PCR reactions.

2.4.1. Color Compensation

In order to detect more than one target sequence in a unique assay, it was necessary to perform a reaction able to analyze simultaneously more than one fluorescent signal. Due to overlap of the emission spectra of the dyes, the known "crosstalk" phenomenon could occur during the emission signal; it happens when one filter combination gathers up signals from a dye measured by another channel. This effect is due to the fact that all fluorescent dyes exhibit emission spectra with long "tails", leading to spectral overlap. Although each emission filter is optimized for a specific emission, the overlapping fluorescence signal may result in misinterpretation of the data. Thus, in order to correct the "crosstalk" effect, the so-called "color compensation" reaction was carried out before performing the multiplex reaction. For this purpose, a separate reaction was prepared using five (5) different mix for the four fluorescent dyes and the negative control (Fig. 2): a minimum of five DNA samples and five negative controls were used to perform the reaction. All the DNA samples gave positive signal for each emission channel. Once saved, the reactions were applied before data analysis, and the software algorithms used the data obtained from the Color Compensation (CC) reaction – when activated – to compensate for the fluorescence "crosstalk".

2.4.2. Multiplex RT-PCR procedure

For the multiplex assay, six larval specimens belonging to each species considered (i.e. *A. simplex* (s. s.), *A. pegreffii, P. decipiens* (s. s.), *P. bulbosa*, *P. krabbei*, *H. aduncum*) were mixed together in order to obtain a homogenate. DNA from this homogenate was then extracted following a normal procedure. Volumes and concentrations of the mix reagents, and the amplification protocol were adjusted and refined with respect to the previously described *singleplex* reactions. In particular, the new mix was performed in 20 µl of final volume including the following reagents: 10 µl of probe master (2×), 0.8 µl of each primer

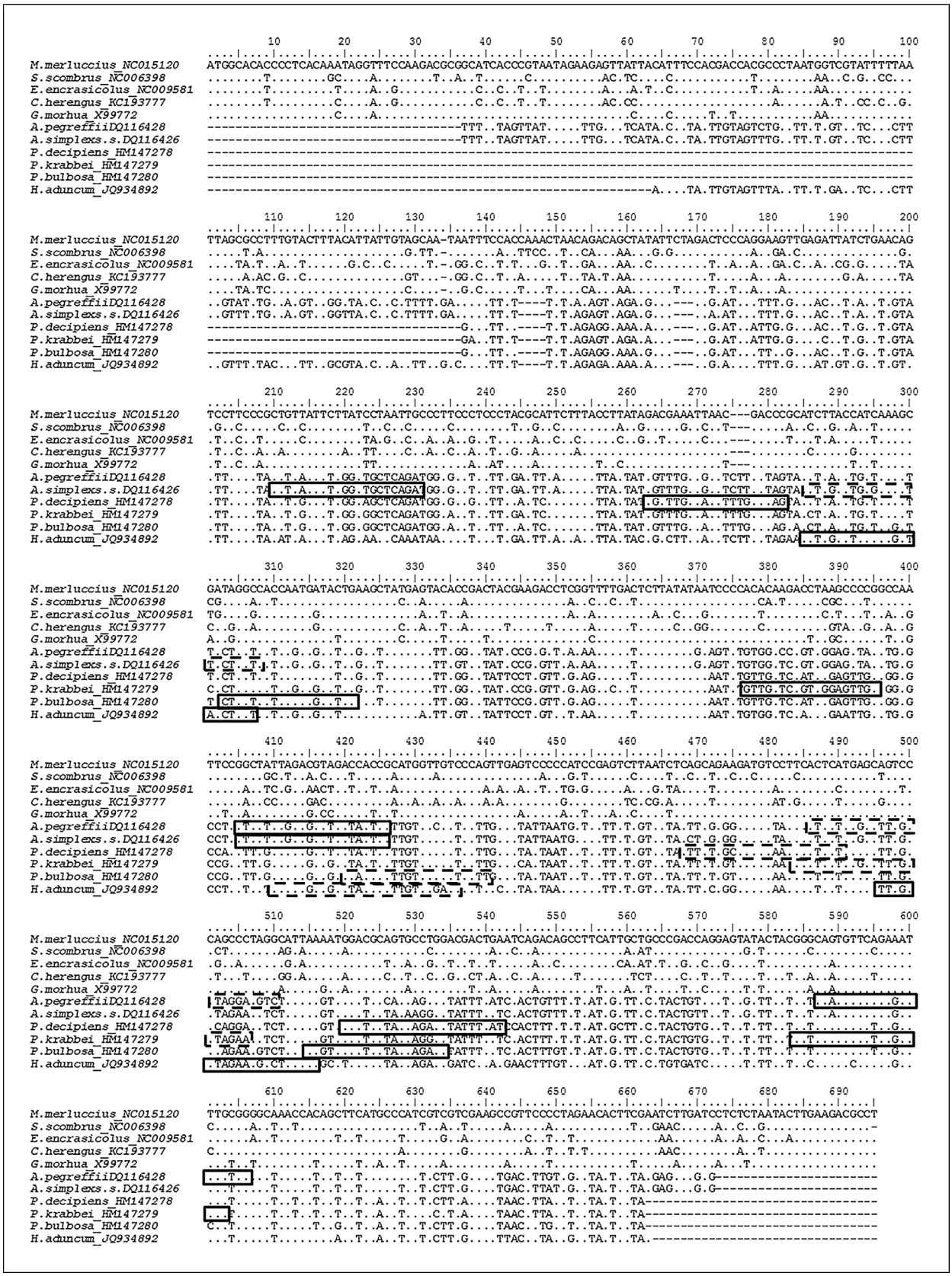

Fig. 1. Spanning nucleotide positions of the newly designed internal primers and probes with respect to original sequences of mtDNA *cox2* locus in species of the genera *Anisakis*, *Pseudoterranova* and *Hysterothylacium*, considered in the present study. Those sequences are shown in comparison with sequences of the same gene locus in fish species of commercial interest found infected by these parasites. Rectangles in black lines indicate the primers nucleotide positions, whereas the dashed lines indicate the nucleotide positions of the probes. For the species *A. simplex* (s. s.) and *H. aduncum*, the probe was designed on the complement strand, in order to have a greater number of C with respect to G (critical condition for an optimal working principle).

Table 2
Internal primers, primers size, spanning nucleotide position, length of fragment obtained, probes sequences and probes labeling, for the species of the genera *Anisakis* (*A. pegreffii* and *A. simplex* (s. s.)), *Pseudoterranova* (*P. decipiens* (s. s.), *P. bulbosa* and *P. krabbei*) and *Hysterothylacium (H. aduncum)*. * = spanning the nucleotide positions with respect to the original mtDNA *cox2* sequences for genus *Anisakis* (629 bp), *Pseudoterranova* (519 bp) and *Hysterothylacium* (593). ** = the probe for *A. simplex* (s. s.) and *H. aduncum* were designed on the complementary strand.

species	internal primers (5′–3′)	Primers size	Spanning nucleotide position*	fragment bp length	probe sequence (5′–3′)	labelling and concentration
A. pegreffii	RTpegF-CTTTTGGAGGTTGATAATCG	20	364–564	200	pegHyPr-CTTGGGCTTTGCCTAGGATGTC	6FAM/BHQ
	RTpegR-CCCACAAATCTCTGAACATT	20				3 nmol
A. simplex (s. s.)	RTsimF-CTTTAATTTTGGTTGCTCAGAT	22	167–383	215	simHyPr-ATGACCAGTGACTTTCACAGTCAAAT**	5-CY/BBQ
	RTsimR-CGATTATCAACCTCCAAAAG	20				3 nmol
P. decipiens (s. s.)	RTdecF-GGCTTGATAAATTTGGACAG	20	120–399	279	decHyPr-CTTCCGGCGATGTAATTCAT	YAK/BHQ
	RTdecR-ATAAAATACCTCTCATAGCATCC	23				3nmol
P. bulbosa	RTbulF-CTGGTCATCARTGGTATTGA	20	160–391	231	bulHyPr-ACAACCGTTGTGTTGTTCCTT	6FAM/BHQ
	RTbulR-CCTCTCATAGCATCCAACTT	20				3 nmol
P. krabbei	RTkraF-GTTGATCAGTTGGAGTTGG	19	234–460	226	kraHyPr-TTCTTGGGCTTTGCCTAGAA	LC640/BHQ or BBQ
	RTkraR-CAAATCTCAGAACACTGACC	20				3nmol
H. aduncum	RThystF-ATTTGACTATCAAGGTAACTGGT	22	215–446	232	hystHyPr-GATCACACAACGGTTATCCACCTCT**	YAK/BHQ
	RThystR-TTAATAGCCATTCTAGGCAAA	21				3nmol

(0.4 μM), 0.4 μl of probe (0.2 μM) specific for each of the parasites species (Table 2), plus 2 μl (~2 ng/μl) of gDNA extracted from the pool of the nematode species tissues; finally, sterilized analytical grade water was added to adjust the volume.

The RT-PCR amplification protocol was adjusted for the multiplex assay as follows: 50 °C for 2 min (activation 1), 95 °C for 10 min (activation 2), followed by 45 cycles at 95 °C for 15 s (annealing), 60 °C for 1 min (extension) and a final step at 40 °C for 30 s (cooling step). The

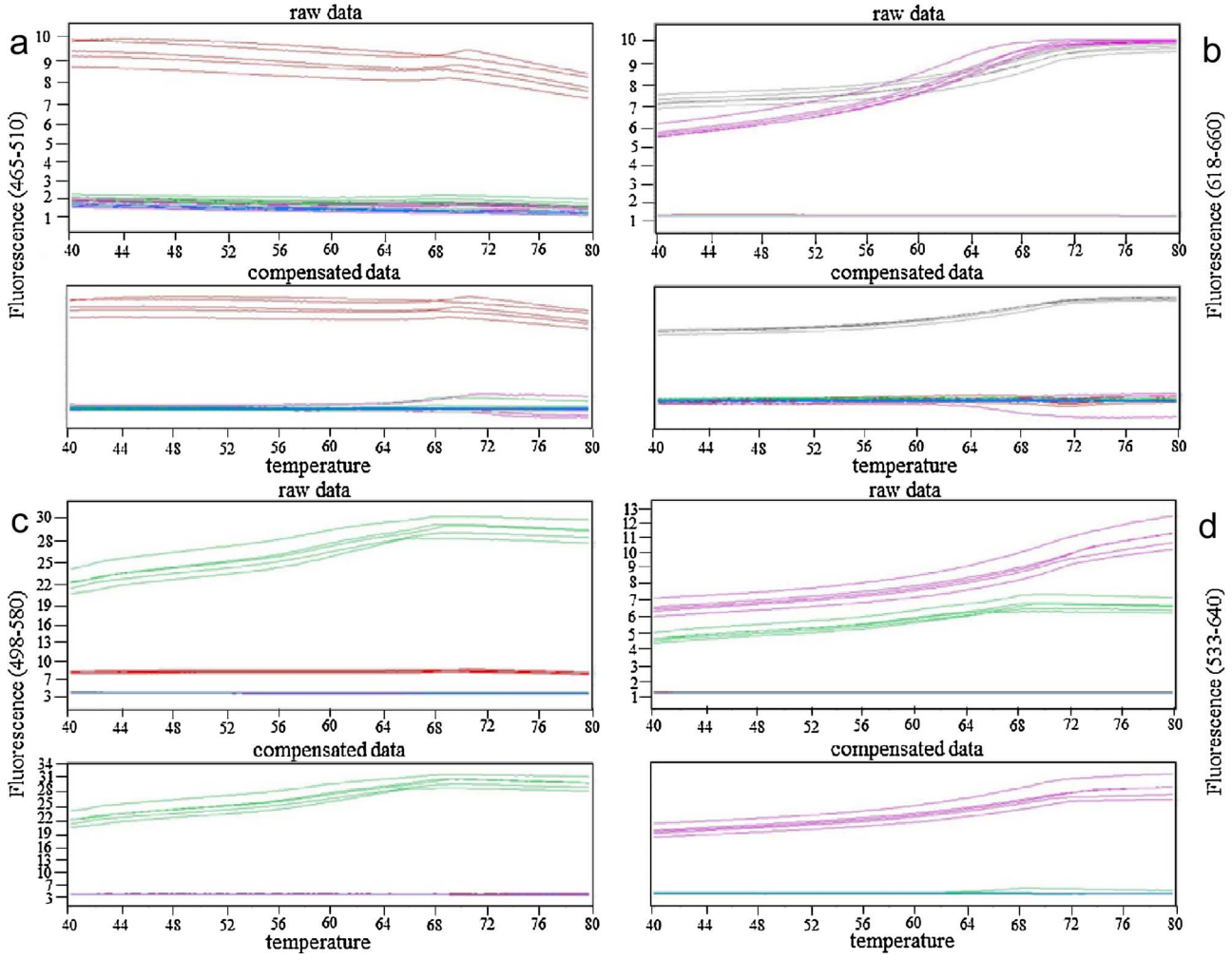

Fig. 2. Color Compensation for the 4 fluorescent dyes used in the experiments: a) 6-FAM (510 nm); b) Cy-5 (660 nm); c) YAK (580 nm); d) LC Red 640 (640 nm). The reaction was carried out using 5 different DNA for each emission channel, and 5 negative controls.

reactions were run in a Light Cycler 480 II System (Roche®) Real Time PCR instrument, using LightCycler 480 Multiwell Plate 96 white (Roche®) and LightCycler 480 Sealing Foil (Roche®).

Primers and probes sequences, fragment length obtained, fluorophore labelling, and concentration of primers and probes used in the multiplex RT-PCR experiments, were the same as those reported in Table 2. Giving the fact that the maximum usage of fluorophores of the RT-PCR instrument used in the present study was four, two species, in pairs (i.e. *A. pegreffii – P. bulbosa; H. aduncum – P. decipiens* (s. s.)) were labeled with the same dye (Table 2). Of course, in order to make visible the specific amplification signal of the DNA from each of the nematode species included in the multiplex reaction, those same pairs of species were labeled with distinct dyes, in a further reaction.

2.5. Standard curve

The efficiency of primers/probe methodology developed in this work was calculated on the base of the standard curve slope obtained by serial dilutions, starting from a template at known concentration (Pfaffl, 2004). In order to perform the standard curve, generally, at least 3 points 5-fold serial DNA dilutions should be prepared for each species. The *slope* of the standard curve describes the kinetics of the PCR amplification; it indicates how quickly the amount of target DNA can be expected to increase with the amplification cycles. The *slope* of the standard curve is also referred to the *Efficiency* (*E*) of the amplification reaction. A perfect amplification reaction would produce a standard curve with efficiency value 2 ($E = 10^{(-1/slope)}$) and a $slope = -3.3$, because the amount of target DNA would double at each amplification cycle (Pfaffl, 2004). The *E* value was automatically calculated by the software of the RT-PCR instrument. The *Error* value (mean squared error of the single data points fit to the regression line) associated to the *slope* and to the *E* values, is a measure of the accuracy of the quantification result based on the standard curve; acceptable value should be < 0.2.

Therefore, standard curves using 6 points of 5-fold dilution series starting from 20 ng/µl (20 ng/µl, 4 ng/µl, 0.8 ng/µl, 0.16 ng/µl, 0.032 ng/µl and 0.006 ng/µl), for each of the six species considered (i.e. *A. simplex* (s. s.), *A. pegreffii, P. decipiens* (s. s.), *P. bulbosa*, *P. krabbei*, *H. aduncum*) were constructed for the fluorescence channels. The last standard curves corresponded to the fluorophore used to label the probes, respectively at 660 nm for *A. simplex* (s. s.), 640 nm for *P. krabbei*, 580 nm for *P. decipiens* (s. s.) and *H. aduncum*, and, finally, 510 nm for both *A. pegreffii* and *P. bulbosa*. In the case of the last two species, the same fluorophore had to be used, due to the limit of four colors of the Roche RT-PCR instrument available during the present study. Threshold Cycle (Ct) data from the standard curves can then be used to convert them from the unknown DNA into concentrations. To make valid the conversions, the amplification efficiencies of the standard curves and unknown DNA samples must be, as much as possible, identical. At least, one sample of known concentration must be included in every experiment. This sample should be designated as a standard and should fall within the range of the standard curve.

2.6. Limit of detection (LOD)

Genomic DNA extracted from the six ascaridoid species, *A. pegreffii*, *A. simplex* (s. s.), *P. decipiens* (s. s.) *P. krabbei, P. bulbosa*, and *H. aduncum* was tested in order to estimate the minimum amount of DNA detectable to obtain a high sensitivity. Sixteen serial 2-fold DNA dilutions from a known amount of DNA were prepared in order to establish the minimum amount of DNA detectable by the system. Dilutions were prepared starting from 20 ng/µl until 0.0006 ng/µl.

3. Results

3.1. Identification of anisakids based on direct DNA sequencing

The mtDNA *cox2* sequences, obtained from the larval *Anisakis* spp. specimens used for the development of RT-PCR primers/probe systems, showed 99% or 100% similarity with sequences of mtDNA *cox2* previously obtained for the species *A. pegreffii* and *A. simplex* (s. s.) (Mattiucci et al., 2014), and retrievable from GenBank with accession numbers, respectively, KC809998 and KC810002. The identification results were confirmed by the diagnostic positions found at the EF1 $\alpha - 1$ region of the nDNA, (i.e. at position 186 and 286, with respect to the 409 bp sequences, referring to the accession numbers KT825684 and KT825685 for *A. pegreffii* and *A. simplex* (s. s.)) (Mattiucci et al., 2016) indicating the specimens belonging to *A. pegreffii* or *A. simplex* (s. s.) in accordance with the mtDNA *cox2* results.

Analogously, the mtDNA *cox2* sequences obtained from the anisakid nematodes belonging to the genus *Pseudoterranova*, used for the development of RT-PCR primers/probe systems, showed 99% or 100% similarity with sequences of mtDNA *cox2* previously obtained and deposited in GenBank for the species *P. decipiens* (s. s.) (accession number HM147278), *P. krabbei* (accession number HM147279) and *P. bulbosa* (accession number HM147280) (Mattiucci et al., 2012). In addition, for the sequences obtained at the ITS1 and ITS2 region of rDNA, the same specimens showed 99% or 100% similarity with sequences of the same gene, previously sequenced and deposited in GenBank with the following accession numbers: *P. decipiens* (s. s.): AJ413968/AJ413967; *P. krabbei*: AJ413965/AJ413966; *P. bulbosa*: AJ413969/AJ413971 (Zhu et al., 2002).

Finally, the sequences of mtDNA *cox2* obtained from the specimens of *H. aduncum* used for the RT-PCR development showed 99% or 100% similarity with sequences of mtDNA *cox2* previously obtained for the species *H. aduncum* and retrievable from GenBank, with accession numbers (JQ934892) (Smrzlić et al., 2012). Additionally, the sequences of *H. aduncum* obtained at the ITS1 and ITS2 region of rDNA showed 99% or 100% similarity with sequences of the same gene, deposited in GenBank with the accession number JX845135 (Haarder et al., 2013).

3.2. Species-specific primers and probes for RT-PCR larval ascaridoid identification

Using the newly designed primers of the mtDNA *cox2,* the following fragment lengths for each anisakid raphidascarid species considered, were obtained: 215 bp for *A. simplex* (s. s.), 200 bp for *A. pegreffii*, 279 bp for *P. decipiens* (s. s.), 226 bp for *P. krabbei,* 231 bp for *A. bulbosa* and, 232 bp for *H. aduncum* (Table 2 and Fig. 1).

3.3. Singleplex RT-PCR results and validation

The aim of the methodological validation was to check if the primers/probe system was able to detect anisakids DNA at specific level. First of all, each probe was tested with pure DNA of the corresponding species, i.e. *A. pegreffii*, *A. simplex* (s. s.), *P. decipiens* (s. s.), *P. bulbosa*, *P. krabbei* and *H. aduncum*. Separated mixes were prepared using species specific probes. The serial dilutions of primers and probes concentration and different annealing temperatures tested for the optimization of RT-PCR reaction conditions have allowed to select the following parameters: 0.2 µM/0.5 µM as optimal primers and probes concentrations, respectively, and 60 °C as the best annealing temperature (T_a).

The results obtained by singleplex RT-PCR reaction in the six distinct anisakid species are reported in Fig. 3. The fluorescent signal is good for all the 6 target species considered (i.e. *A. pegreffii*, *A. simplex* (s. s.), *P. decipiens* (s. s.), *P. bulbosa*, *P. krabbei* and *H. aduncum*). The fluorescent signal for the species *P. decipiens* (s. s.) and *H. aduncum* have been found slightly weaker due probably to the low amount of DNA obtained. Anyhow, PCR products were sequenced to confirm the species

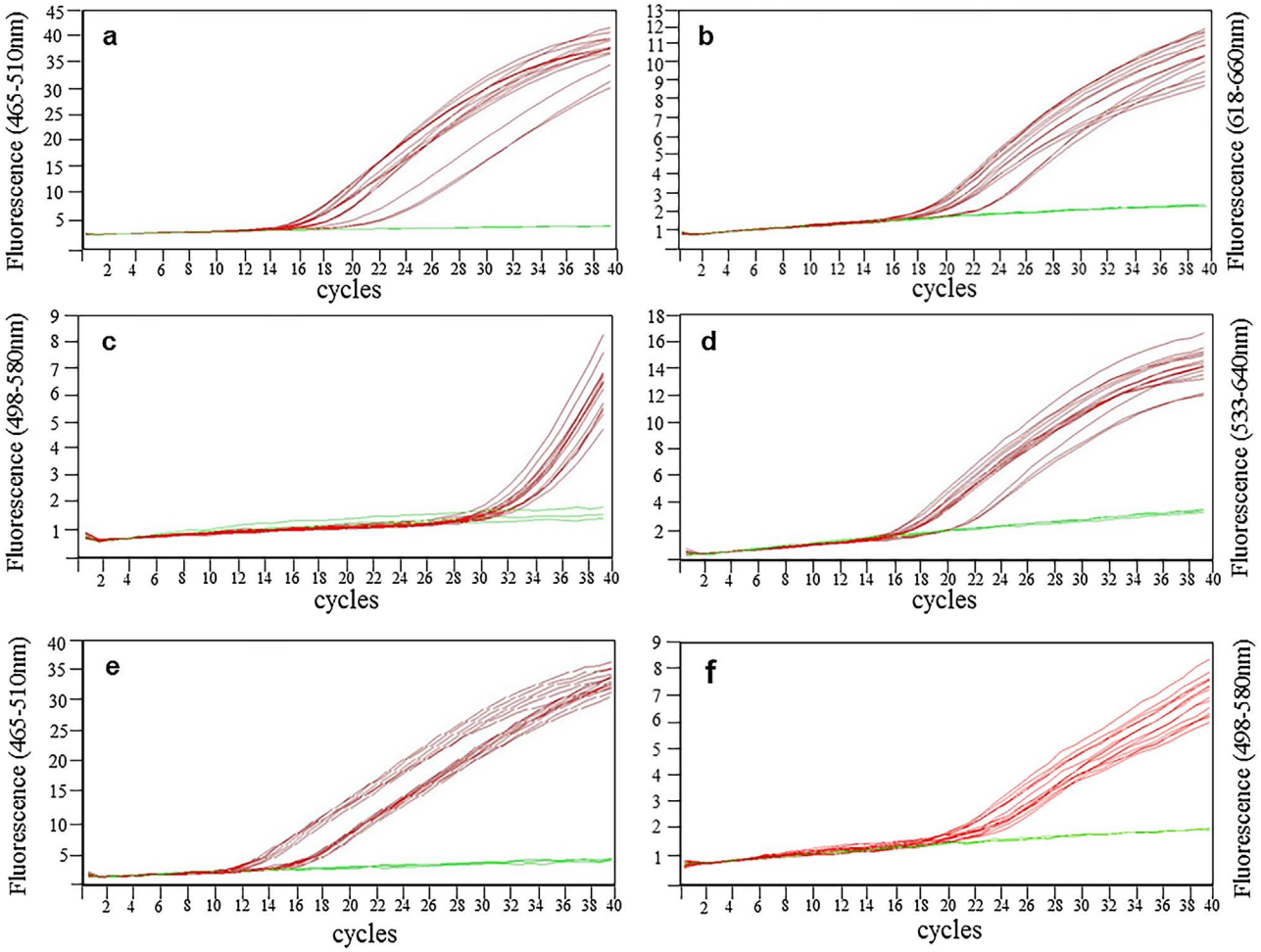

Fig. 3. Probes validation in *singleplex* reactions of the 6 ascaridoid species: a) *Anisakis pegreffii*; b) *A. simplex* (s. s.); c) *Pseudoterranova decipiens* (s. s.); d) *P. krabbei*; e) *P. bulbosa*; f) *Hysterothylacium aduncum*. The reactions were performed in triplicate on 6 different DNA extractions.

identification, and BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis showed the 99–100% match to species targeted by the assay.

3.4. Standard curve

The amplification plot for the nematode species considered generated a slope with values between −3.31 and −3.07; whereas, the PCR efficiency values varied between 2.015 and 2.11, for the same species (data not shown). The error values varied between 0.045 and 0.049.

3.5. Specificity, sensitivity and limit of detection (LOD)

The specificity of primers/probe combination system was confirmed with a double test: all the specimens examined were directly sequenced and identified to species level by different genes, to confirm their specific identification by primers/probe sets; no cross-reactivity was observed with DNA of several fish species tested using those anisakids primers/probe sets developed (data not shown).

Highest specificity and sensitivity of the method was obtained at annealing temperature of 60 °C and performing the RT-PCR reaction with 0.2 μM and 0.5 μM primers and probes as optimal concentration.

Generally, in the RT-PCR assay, the highest sensitivity is represented by the earlier Threshold Cycle (C_t) value, or rather by the cycle number at which the amount of amplified template reaches a fixed threshold. In this regard, the C_t parameter is a very important factor; it represents a relative measure of the concentration of target DNA in the PCR reaction. A rise in C_t value is associated with a decrease in DNA amount; it is inversely correlated to the logarithm of initial DNA concentration. The intersection of the C_t value with the standard curve, gives the amount of DNA in the reaction and the relative DNA concentration.

Genomic DNA extracted from the ascaridoid species, *A. pegreffii, A. simplex* (s. s.), *P. decipiens* (s. s.), *P. bulbosa*, *P. krabbei,* and *H. aduncum* was tested in order to estimate the minimum amount of DNA detectable to obtain a high sensitivity. 16 Serial 2-fold DNA dilutions, starting from 20 ng/μl, were prepared, in order to establish the minimum amount of DNA detectable by the system. The detection limit was established lower than 0.0006 ng/μl (Fig. 4).

3.6. RT- PCR multiplex results and validation

Multiplex RT-PCR reactions were performed using different primers and probes combinations, varying concentrations and volumes. The amount of target DNA has been changed for different multiplex reactions.

The best result was obtained adjusting and refining the reaction mix and the run temperature protocol, as described above (see Section 2.4.2). Good fluorescence signal was obtained starting from the simultaneous DNA extraction of a single larval specimen from each of the six ascaridoid species by the best balance between the primers and probes concentration; the optimal concentrations of primers/probes was 0.4 and 0.2 μM, respectively. The results obtained by the RT-PCR multiplex reaction are reported in Fig. 5a, b, c, d, e, and f respectively, for *A. pegreffii, A. simplex* (s. s.), *P. decipiens* (s. s.), *P. krabbei*, *P. bulbosa*

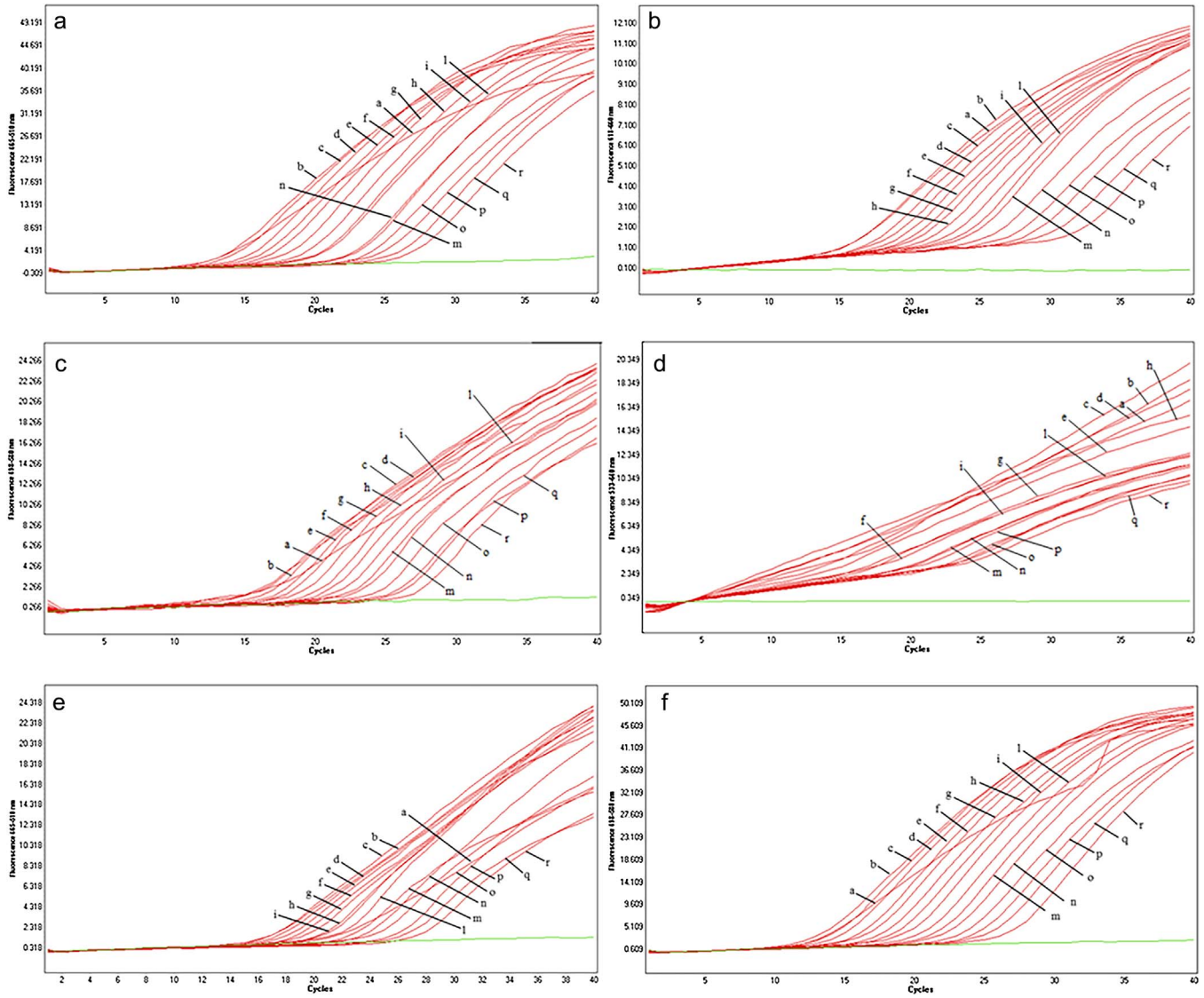

Fig. 4. Limit Of Detection (LOD) for the parasite species under study (i.e. a) *A. pegreffii,* b) *A. simplex* (s. s.), c) *P. decipiens* (s. s.), d) *P. krabbei,* e) *P. bulbosa* and f) *H. aduncum*). The reactions were carried out starting from 20 ng/μl of total DNA, and 16 serial 2-fold DNA dilutions were prepared. The detection limit was established at a value lower than 0.0006 ng/μl for the six species considered. Serial dilutions for each species' LOD reactions are indicated in the figure as follow: a = 20 ng/μl; b = 10 ng/μl; c = 5 ng/μl; d = 2.5 ng/μl; e = 1.25 ng/μl; f = 0.62 ng/μl; g = 0.31 ng/μl; h = 0.15 ng/μl; i = 0.08 ng/μl; l = 0.04 ng/μl; m = 0.02 ng/μl; n = 0.01 ng/μl; o = 0.005 ng/μl; p = 0.0025; q = 0.0012 ng/μl; r = 0.0006 ng/μl.

and *H. aduncum*. Being four species (i.e *A. pegreffii – P. bulbosa; P. decipiens* (s. s.) *– H. aduncum*) previously labelled in pairs with the same fluorophore, consequently the discrimination between those species was obtained by further RT-PCR reactions (see Section 2.4.2); in each further reaction, the two species were labeled with different dyes (Fig. 5).

4. Discussion

One of the most interesting aspects of RT-PCR, based on detection of fluorophoric labeled oligonucleotides such as hydrolysis probes, is the possibility to detect conveniently multiple DNA targets in the same PCR reaction (multiplex PCR). Ideally, a multiplex RT-PCR should be able to detect and differentiate many different targets without any "cross-talk", i.e. the detection of a single target should not influence the detection of another one, without loss of sensitivity.

In this study, RT-PCR primers-probes assays based on the mitochondrial gene mtDNA *cox2* were established for the identification of two species of *Anisakis* (i.e., *A. pegreffii* and *A. simplex* (s. s.)), three species of *Pseudoterranova* (i.e., *P. decipiens* (s. s.), *P. krabbei* and *P. bulbosa*), and one of *Hysterothylacium* (i.e. *H. aduncum*).

The application of molecular markers from mitochondrial gene increases the amplification yield, due to hundreds of thousand copies of mitochondrial genome, provided that the initial number of copies in the template is very high. In addition, the mitochondrial genome is more stable than the nuclear one (Unseld et al., 1995; Lopez and Pardo, 2010). Another critical factor for adequate design of primers and probe is the detection of a particular DNA region with low to absent gene variation, in order to identify conserved nucleotide positions, able to discriminate between the distinct species. In this respect, mtDNA *cox2* resulted to be a good candidate, constantly used for sequencing analysis in the identification of species of the genera *Anisakis* (Mattiucci et al., 2014, 2017), *Pseudoterranova* (Timi et al., 2014) and *Hysterothylacium* (Smrzlić et al., 2012) species, and also for its high genetic variability so far observed in those genera (Mattiucci et al., 2014; Timi et al., 2014). Moreover, several mtDNA *cox2* sequences, concerning those nematode species considered in this study, are available in the genetic data Banks.

The RT-PCR probes based on the mtDNA *cox2* are developed in order to recognize and quantify DNA belonging to five ascaridoid species, and to be used both in singleplex and multiplex reactions.

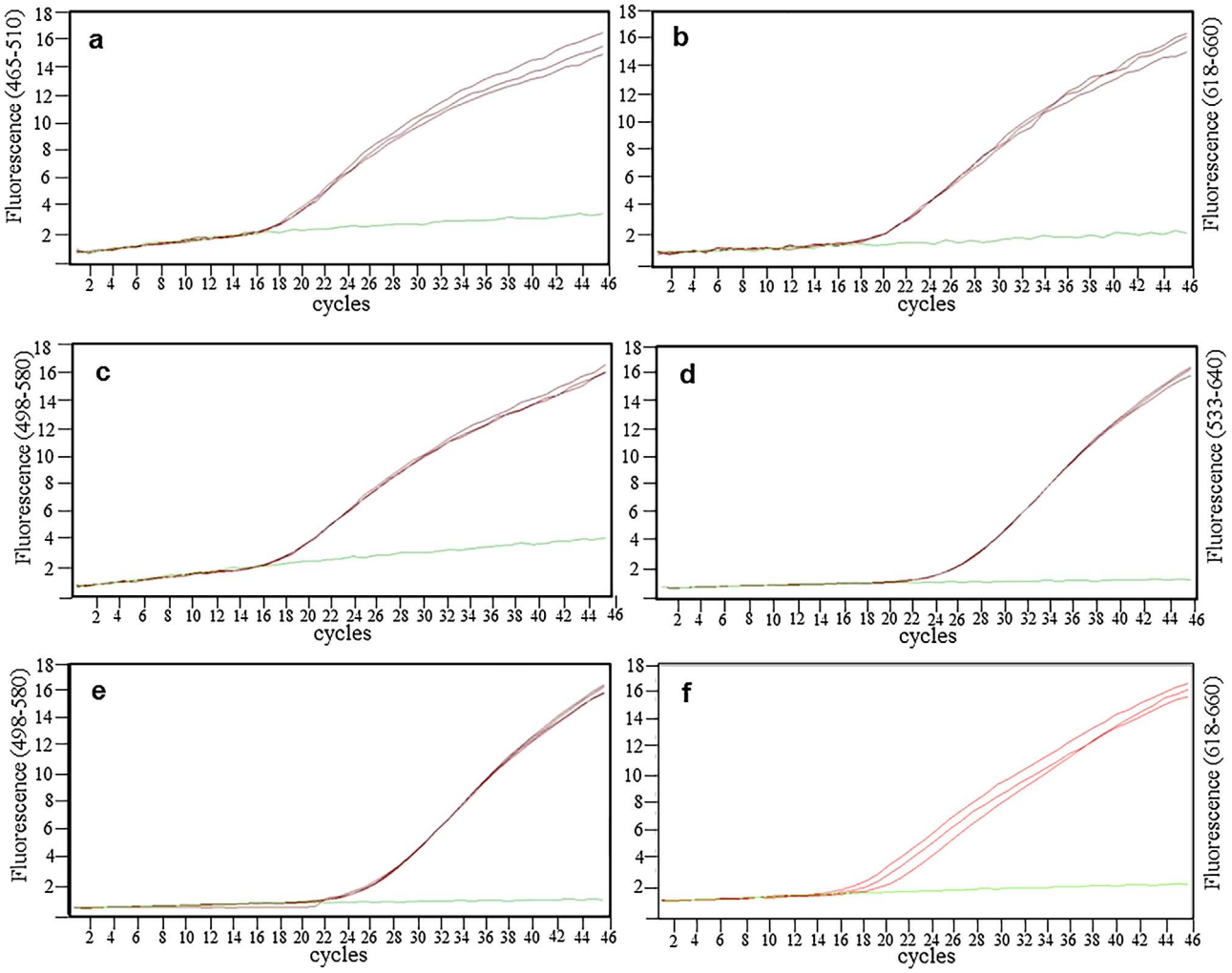

Fig. 5. Probes validation in multiplex reactions for six ascaridoid species under studied: a) *Anisakis pegreffii*, b) *A. simplex* (s. s.), c) *Pseudoterranova decipiens* (s. s.), d) *P. krabbei*, e) *P. bulbosa* and f) *Hysterothylacium aduncum*. Simultaneous DNA extractions were performed. The DNA was obtained from a single larval specimen of each of thesix species tested. The RT-PCR reactions were performed in triplicate. The six screens obtained for each of the six anisakid species in the same multiplex reaction are shown.

Although several attempts to develop primers/probes to be used in RT-PCR methodology for the identification and detection of anisakids were proposed (Espiñeira et al., 2010; Mossali et al., 2010; Fang et al., 2011; Herrero et al., 2011), a species-specific RT-PCR protocol for the simultaneous detection of the most important zoonotic species present in fish of commercial value in European waters has hitherto been lacking. In addition, most of the attempts to design a protocol based on RT-PCR have been based on the ITS region of rDNA, for instance on the two *Anisakis* species *A. simplex* (s. s.) and *A. pegreffii*. However, this locus has been recently considered not able, as a stand alone diagnostic marker, to discriminate completely between these two species (Mattiucci et al., 2016); as a consequence, it could lack of specificity in those species-specific detection.

The herein proposed RT-PCR method, based on the mtDNA *cox2*, matches the requirements for highest specificity, sensitivity and reproducibility of the assay. Indeed, two protocols (singleplex and multiplex) were assessed, and the results indicate good performance in the correct identification of the anisakid samples used for the protocol development and validation. On the other hand, as we have also demonstrated, the RT-PCR probe doesn't cross-react with the DNA of the fish species infected by those parasite species (no cross-reaction). Therefore, the proposed method matches the characteristic of the specificity. The other characteristic required in the validation method includes the sensitivity. In RT-PCR methodology the highest sensitivity corresponds to the lowest amount of DNA detectable in the sample tested. Serial dilutions starting from a known amount of DNA were performed in order to detect the lowest concentration of DNA detectable with primers/probe systems developed. The primers/probe RT-PCR systems proposed for the five species of those genera allowed the detection limit lower than 0.0006 ng/µl of DNA.

5. Concluding remarks

From a practical point of view, the main advantage of the use of RT-PCR probes, with respect to the direct sequencing of target gene loci, lies in the fact that sequence analysis is generally time consuming, especially when large numbers of worms need to be identified. Therefore, hydrolysis probes used in a single RT-PCR reaction (multiplex reaction), such as the present one, allow rapid identification of several ascaridoid individuals. It could be used in routine quality inspections, e.g. by the fish processing industry, in national public health laboratories, or in reference centers for the diagnosis of anisakids, either from fish or from human samples. The developed method will allow, in the future, also the DNA identification of those anisakid species of the genera *Anisakis* and *Pseudoterranova,* to be distinguished from species of the genus *Hysterothylacium* occurring in fresh fish. In fact, the protocol for the hydrolysis probes established in this study, validated over several specimens belonging to species of those genera studied, has the potential to be used also for the direct detection of the parasites' DNA in fish fillets, and other "seafood" products, such as

those frozen and canned. Indeed, when infecting at high prevalence and density a fish species, ascaridoid nematodes have an adverse effect on fisheries (Levsen et al., 2017, in press). Thus, the rapid identification of those zoonotic parasites in wild fish species from European waters acquires much importance also from an epidemiological point of view.

In addition, the method could be also used in the rapid, sensible and specific identification, in laboratory of clinical Hospitals, of those species so far known as etiological agent of human anisakidosis. In this respect, the RT-PCR probe assay could be used in the molecular detection of anisakid DNA, even at very low quantity, and successfully diagnose the presence of those parasites in gastric or intestinal biopsy and from surgically removed granulomas from humans (Mattiucci et al., submitted).

Authors' contributions

MP, SM, AL, MG and GN planned the work. MP, SM, AC and MG performed the molecular identification of the parasites, and the RT-PCR assays. MP, SM reviewed the literature and wrote the MS. AL and MG have also provided some samples used for the experiments. All the authors have reviewed the MS, and approved its content.

Declaration of interest

The authors declare that they have no competing interests.

Acknowledgements

The study was supported by funding from the European Union's Seventh Framework Programme (FP7) for research, technological development and demonstration, under grant agreement n. 312068 "PARASITE" and by Grant (2015) from "Sapienza - University of Rome" (n. C26H15SBJ4). The authors thank the anonymous Referees, whose suggestions and comments were useful to improve the manuscript.

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